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(54) Title: METHOD FOR THE PRODUCTION AND USE OF DENDRITIC CELLS

(57) Abstract

The invention is directed to compositions and methods for the preparation of a composition containing conditioned medium that enhances the growth of antigen presenting cells in culture. Compositions are specifically useful for the growth and maintenance of dendritic cells, macrophages, and monocytes. Methods comprise culturing a population of cells in a composition containing a conditioned medium prepared according to the methods of the invention. Cultured cells can be further treated with compounds which reduce, suppress or eliminate the growth of undesired cell types, such as T–cells. Cells that proliferate according to these methods comprise enriched populations of dendritic cells, monocytes and macrophages. Such cells are useful in the creation of cellular vaccines and for immunotherapy, and can serve as useful vehicles for gene therapy.

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METHODS FOR THE PRODUCTION AND USE OF DENDRITIC CELLS

Field of the Invention

The present invention relates to methods for the enrichment and culture of dendritic cells, monocytes, and macrophages (hereinafter "antigen presenting cells") utilizing a starting cell sample and a conditioned medium that favors growth and survival of these cells. Cells produced by these methods may be used *in vitro* to generate antigen-specific T cells for use in the treatment of cancer, infectious diseases and other disorders, *in vivo* as a therapeutic or prophylactic cellular vaccine or as a cellular vehicle to carry genetic material to a host for genetic therapy. The invention also relates to novel compositions containing conditioned medium and novel methods for the production of such conditioned medium, as well as a novel process for the suppression of T-cell growth in culture.

Background of the Invention

Dendritic cells are the most effective professional antigen presenting cells of the immune system. Macrophages are phagocytic antigen-presenting cells; however, they must be activated prior to expressing cell surface receptors necessary for effective antigen presentation. Monocytes are circulating mononuclear phagocytic leukocytes which eventually differentiate further to form macrophages or dendritic cells, depending on the cytokine environment. It is not currently clear whether monocyte-derived dendritic cells and macrophages are terminally differentiated, or whether interconversion between these cell types is possible. An effective cell-mediated immune response is predicated upon the ability of T lymphocytes to mount a specific attack against non-self while remaining tolerant or inactive to self. Non-self includes pathogenic foreign microorganisms such as viruses, bacteria and parasites, allogeneic tissue transplants, as well as transformed cells expressing molecules not normally present on healthy tissue. Specific, non-self moieties recognized by T lymphocytes are small peptide fragments of foreign antigens. For T lymphocytes to recognize and respond to an antigen, the antigen-derived peptides must be presented on the surface of an antigen presenting cell (APC) in association with a class I or class II major

histocompatibility complex (MHC) molecule.

The recognition structure on the APC is composed of a small portion of the antigen (the antigenic peptide) attached to the peptide binding cleft of the MHC molecule. The antigen recognition element of T cells is a surface membrane protein of diverse antigenic specificity, known as the T cell receptor (TcR). TcR:MHC recognition normally invokes T cell activation that, depending upon the type of T cell activated, leads to cell proliferation, cytokine secretion or cytotoxic activity directed at cells bearing the target antigen. In general, $CD4^+$ T lymphocytes, most of which have a helper function, recognize antigenic peptides derived from exogenous foreign antigens that are taken up and processed by the APC and presented in association with class II MHC molecules. By contrast, $CD8^+$ T lymphocytes, most of which have a cytolytic function (CTL), recognize and kill host cells presenting antigenic peptides derived from endogenously synthesized foreign antigens in association with class I MHC molecules which are present on all host cells. Normally, for recognition of a foreign antigenic peptide to occur, the T lymphocyte and the APC must be from the same individual, a phenomenon known as MHC restriction. One artificial exception is the mixed leukocyte reaction in which the T cells of one individual recognize and react to the allogenic MHC molecules on the APC of another individual. Dendritic cells express very high levels of both class I and class II MHC molecules and for this reason, they are the strongest stimulators of the allogeneic mixed leukocyte reaction.

Due to the simultaneous expression of both class I and class II MHC, and of several co-stimulatory molecules necessary for full T cell activation, dendritic cells are extremely potent activators of both $CD8^+$ CTLs and $CD4^+$ T helper cells. Although other antigen presenting cells are also capable of stimulating activated T cells or memory T cells (*i.e.* T cells primed through a previous encounter with antigen), only dendritic cells can stimulate naive, unprimed T cells in an antigen specific manner. Thus, as the professional antigen presenting cells, dendritic cells occupy a central and pivotal position in the immune system.

T lymphocytes are bone marrow derived cells that differentiate in the

thymus and are educated to distinguish self antigens from non-self antigens. Functionally, there are two types of T cells, the helper T cell and the cytotoxic T cell. When stimulated by the presentation of a foreign antigen by an APC, $CD4^+$ helper T cells respond by secreting cytokines. Cytokines are soluble protein molecules that exert regulatory effects on other cell types, including cytotoxic T cells, other helper T cells, B cells and macrophages. Thus, helper T cells exert control over many types of processes carried out by these cells, such as T cell-mediated killing, antibody production and inflammation. The main function of $CD8^+$ cytotoxic T cells is to destroy host cells that have been infected by virus or other infectious agents, or that have undergone malignant transformation. Cytotoxic T cells may also secrete cytokines and exert regulatory effects on other cells of the immune system.

While the normal response to T-cell receptor - antigen - MHC binding is for the T-cell to become activated, it is possible to alter the antigen, the culture conditions (*in vitro*) or the cell surface markers on dendritic cells themselves, in order to induce incomplete T-cell activation, or to induce T-cell unresponsiveness to the presented antigen. This can be useful when a patient suffers from an improper or excessive immune response to a particular antigen and down-regulation of the immune response is desirable.

Where a peptide ligand corresponding to a proteinaceous antigen of concern has been identified and sequenced, it is possible to generate altered peptide ligands differing in only one or a very few amino acids which will bind the MHC molecule on the dendritic cell normally, but will have an altered interaction with the T-cell receptor, resulting either in only partial activation of the T-cell, or antagonism of the T-cell receptor so as to delay or prevent subsequent activation of that T-cell. Thus, dendritic cells or macrophages produced by the methods of the invention can be exposed to altered peptide ligands *in vitro* and subsequently re-introduced into a patient with the same or a compatible MHC haplotype in order to down-regulate the T-cell response to the antigen of concern.

T-cell activation in response to antigen- MHC binding is dependent on a

co-stimulatory signal between the antigen-presenting cell and the T-cell. One example of such a co-stimulatory signal is the signal generated by the interaction between B7.1 or 7.2 on dendritic cells, and CD 28 on T-helper cells. This interaction, in combination with the antigen-MHC-TcR interaction, stimulates the T-helper cell to enter the G_1 phase of the cell cycle, and induces transcription of the gene for IL-2 and the α chain of the high affinity IL-2 receptor. Where the transmission of the co-stimulatory signal is blocked, for example by interference with the interaction between B7 and CD28, the T-cell which binds the antigen-MHC complex will enter into a state of non-responsiveness called clonal anergy and will not proliferate in response to an antigen-MHC complex.

It is desirable to induce clonal anergy in situations where a patient suffers from an excessive cellular immune response, and down-regulation of this response is needed. One means of achieving this *in vitro* is to inactivate B7.1 or B7.2 genes or their gene products in dendritic cells or macrophages of the same haplotype as the patient produced by the methods of the invention, followed by the introduction of the altered dendritic cells or macrophages into the patient. For example, it is possible to introduce an insertion mutation into the coding region of the B7.1 and B7.2 genes, thereby altering their conformation and preventing effective interaction with CD28. Alternatively, a frame-shift mutation could be introduced into the coding region of the B7.1 or B7.2 genes to generate non-functional protein products. A further means to prevent or reduce the B7-CD28 interaction is to prevent or reduce the expression of the B7.1 or B 7.2 genes in dendritic cells, for example, by mutating the promoter region of the gene or the ribosome-binding site in the corresponding RNA sequence. Several B7 mRNA's have been sequenced, and mutant B7 proteins have been reported in the art. Thus, it is well within the ability of one skilled in the art to prepare non-functional B7 mutant dendritic cells or macrophages in culture.

Various tissues have been used as sources of dendritic cells, including peripheral blood, bone marrow and enriched $CD34^+$ progenitor cells, and many techniques have been used to enrich dendritic cells from these sources, including density gradient centrifugation, centrifugal elutriation, differential adherence to plastic and immuno-affinity selection techniques employing antibodies specific for dendritic cell

surface markers. To enrich dendritic cells, the positive selection immuno-affinity techniques have employed antibodies specific for markers that are present on dendritic cells, such as CD83, CD4 and class II MHC, while the negative selection immuno-affinity techniques have used antibodies specific for markers that are not present on dendritic cells, such as CD3 and CD14. Many cytokines have been used to culture dendritic cells including various combinations of GM-CSF, IL-4, TNF-α, IL-13, SCF, IL-6, IL-3, TGF-β, Flt-3 ligand and erythropoietin. There is some controversy as to the exact phenotype of dendritic cells, but most reports agree that while dendritic cells bear high levels of class I and class II MHC molecules in addition to the co-stimulatory molecules, CD80 (B7.1) and CD86 (B7.2), mature dendritic cells lack the lineage specific markers CD3 (T cells), CD19 (B cells), CD14 (monocytes and macrophages), and CD56 (natural killer cells).

CD83 has recently been described as a specific marker of dendritic cells (Blood 89:3708, 1997). Phenotype notwithstanding, the definitive identification of dendritic cells depends on a functional analysis of the cells: (1) dendritic cells are the most potent stimulators of the allogeneic mixed leukocyte reaction, (2) dendritic cells are very potent antigen presenting cells for primed T cells, and (3) dendritic cells are uniquely able to present antigen to unprimed T cells.

Conventional methods of dendritic cell enrichment and culture are complex, requiring specialized techniques and expensive growth factors. Yields are often low and do not significantly increase dendritic cell numbers beyond the starting number. Therefore, a need has arisen to produce large numbers of dendritic cells in a short time and at a low cost using a relatively small sample.

Summary of the Invention

The present invention specifically reduces the problems and disadvantages associated with current strategies and designs and provides new compositions and methods for the production and use of dendritic cells, macrophages, and monocytes.

One embodiment of the invention is directed to methods for the production of a conditioned medium "DSCM" that stimulates the growth of dendritic cells, monocytes, and macrophages. These methods comprise culturing of a population of cells of the lympho-hematopoietic system in the presence of one or more stimulatory biochemical agents that stimulate the secretion of a selection of cytokines into the medium.

Another embodiment of the invention is directed to compositions that stimulate the growth of dendritic cells, monocytes, and macrophages. Compositions comprise conditioned media produced by the methods of the invention. These compositions stimulate the growth of dendritic cells, monocytes, and macrophages in culture.

Another embodiment of the invention is directed to methods for the culture of preselected antigen presenting cell types or progenitors thereof in order to increase their number or their relative proportion in the culture relative to the starting cell population. A starting population containing viable antigen presenting cells or progenitors thereof is cultured in a culture medium containing conditioned medium DSCM produced according to the methods of the invention that favors the growth of the preselected cell type or types or progenitors thereof. Cultures may further contain a compound effective to suppress, delay or inhibit the growth of undesired cell types. Where the undesired cell type is T-cells, a preferred embodiment of the invention is the use of serum, plasma, anti-IL-2 antibodies as a compound to suppress, delay, or inhibit the growth of T-cells. It is also a preferred embodiment of this invention where the undesired cell type is T-cells to use immunosuppressive agents such as cyclosporin, FK 506, or rapamycin as compounds to suppress, delay, or inhibit the growth of T-cells. Where the undesired cell type is macrophages, a preferred embodiment of the invention is to use antibody to macrophage colony stimulating factor to suppress, delay, or inhibit the growth of macrophages.

Another embodiment of the of the invention is directed to the methods

for inducing the differentiation of mulitpotential hematopoietic progenitor cells into preselected antigen presenting cells by culturing the multipotential hematopoietic progenitor cells in the conditioned medium DSCM. Cultures may further contain a compound effective to suppress, delay or inhibit the growth of undesired cell types, including T-cells where appropriate. A preferred embodiment of the invention is directed to methods for inducing the differentiation of mulitpotential hematopoietic progenitor cells into preselected antigen presenting cells where the starting population is enriched in CD 34+ cells.

Another embodiment of the invention is directed to a method of suppressing T-cell growth in culture by culturing a cell population containing T-cells or their progenitors in a culture medium which contains an effective amount of a T-cell growth-suppressive compound. A preferred embodiment of the invention is directed to a method of suppressing T-cell growth in culture by culturing a cell population containing T-cells or their progenitors in a culture medium which contains a T-cell growth suppressive compound selected from serum, plasma, anti-IL-2 antibody, or an immunosuppressive agent such as cyclosporin A, FK 506, and rapamycin.

Another embodiment of the invention is directed to methods for utilizing antigen presenting cells produced according to the methods of the invention. These cells can be used in therapy and prophylaxis to treat or prevent disorders by, for example, adoptive immunotherapy, gene transfer and other methods.

Dendritic cells and macrophages produced according to the methods of the invention may be treated with an antigen of interest in order to induce the dendritic cells or macrophages to process the antigen and present antigenic fragments for recognition by T-cells having the same or sufficiently similar MHC haplotype as the dendritic cells or macrophages. The dendritic cells or macrophages presenting the desired antigen may then either be introduced into the patient to activate T-cells *in vivo*, or they may be cultured with T-cells *in vitro*, and the resultant activated T-cells may be introduced into the patient. Depending on the desired outcome, the antigen to which the cells are exposed and other culture conditions may be selected so that upon T-cell

receptor binding to the antigen-MHC complex, the T-cell is either: (a) activated in the normal manner, (b) partially activated, or (c) rendered non-responsive to the antigen.

Antigen presenting cells produced according to the methods of the invention may be transfected with genetic material encoding a protein of interest. The purpose of such transfection may be either: (a) to induce the presentation of the protein product of the introduced genetic material in association with class I MHC molecules in order to induce T-cell activation; or (b) to induce the expression of the protein product of the genetic material in the patient in order to regulate or supplement some aspect of that patient's endogenous biological processes.

Another embodiment of the invention is directed to a population of preselected antigen presenting cells produced according to the methods of the invention. Cells can be grown and maintained in mixed or substantially pure populations. A preferred embodiment of the invention is a population of dendritic cells produced by the methods of the invention. A more preferred embodiment of the invention is a population of dendritic cells produced by the methods of the invention and substantially free of viable cells of any other type. Another preferred embodiment of the invention is a population of macrophages produced by the methods of the invention. A more preferred embodiment is a population of macrophages produced by the methods of the invention and substantially free of viable cells of any other type. Another preferred embodiment of the invention is a population of monocytes produced by the methods of the invention.

Other embodiments and advantages of the invention are set forth, in part, in the description which follows and, in part, will be obvious from this description or may be learned from the practice of the invention.

Description of the Drawings

Figure 1 - Two photographs of human blood LDMNC cultured in the presence of 20% umbilical cord blood plasma (a) before and (b) after treatment with

dendritic cell selective medium ("DSCM") as described in Example 6 below. Magnification = 200X.

Figure 2 - Histograms from flow cytometric analyses of human blood low density mononuclear cells ("LDMNC") cultured in the presence of umbilical cord blood plasma (a) before and (b) after treatment with DSCM, as described in Example 6 below. Cells were stained with a specific monoclonal antibody to CD 83 (black line), or with an isotype-matched control antibody of irrelevant specificity (grey line).

Figure 3 - Bar chart showing the proliferative response, measured by ^{3}H-thymidine uptake (cpm), of autologous and allogeneic responder LDMNC to stimulation with DSCM-generated dendritic cells in the mixed leukocyte reaction, as described in Example 7 below. (5% P - DC: dendritic cells derived using 5% umbilical cord blood plasma, 20% P - DC: dendritic cells derived using 20% umbilical cord blood plasma).

Figure 4 - Bar charts from two separate experiments (Example 8, below) illustrating the ability of DSCM-generated dendritic cells to present foreign antigen to T cells. The proliferative response, measured by ^{3}H-thymidine uptake (cpm), of (a) autologous LDMNC responder cells (containing T cells) and (b) purified autologous T cells, to presentation of tetanus toxoid antigen by DSCM-generated dendritic cells is shown.

Description of the Invention

As embodied and broadly described herein, the present invention is directed to compositions for the growth of dendritic cells, macrophages, and monocytes (herein collectively "antigen presenting cells"), to methods for the production of such compositions, to methods for culturing antigen presenting cells, to antigen presenting cell populations produced by these methods and to methods for the use of these antigen presenting cells.

Conventional methods of dendritic cell enrichment as well as culturing

from cell samples are fairly complex requiring specialized techniques and expensive mixtures of growth factors. These techniques are labor intensive and carry a significant cost. Even with these techniques, cell numbers are often low and do not significantly increase beyond the initial amount after extended culturing. More recent techniques have focused on the physical selection of dendritic cells from mixed populations using antibodies to cell dendritic cell surface markers, fluorescent activated cell sorting (FACS) or immunoaffinity procedures. These techniques also suffer from low yields, high cell death rates, an inability to ensure selection of only dendritic cells and an inability to rapidly expand the few selected cells that survive the procedure.

The present invention provides new methods and novel compositions for the enrichment and also culture of dendritic cells. Using these compositions and methods, large numbers of dendritic cells can be easily, inexpensively and rapidly produced from a relatively small sample of cells. These methods are straightforward and overcome the need for extensive cell separation and purification. Enrichment and subsequent culture of dendritic cells is achieved through the use of a type of conditioned medium. The conditioned medium, referred to herein as dendritic cell selective conditioned medium (DSCM), is also a potent stimulator of T lymphocyte growth and maintenance. Surprisingly, it was discovered that by suppressing this T cell expansion activity, a second activity is revealed. This second activity allows for the enrichment and culture of cells with the morphologic, phenotypic and functional characteristics of dendritic cells. Such methods and culture conditions allow for the rapid expansion of large numbers of dendritic cells from a diverse cell background, such as a sample of blood cells, and the culturing of such cells for manipulation and later use.

A further aspect of the invention is the dose-dependent inhibition of T cells by human umbilical cord blood plasma. Suppression of T cell expansion activity of DSCM may be achieved by culturing low density mononuclear cells (LDMNC) from human blood in the absence of exogenous cytokines using relatively high concentrations (5 - 20% or greater) of umbilical cord blood plasma for several days or weeks. T cell suppression may be further enhanced through the depletion of T cells from the LDMNC prior to culture in umbilical cord blood plasma. By way of example, T cells may be

depleted by affinity depletion of $CD3^+$ cells using a specific antibody and magnetic beads. The addition of 5% DSCM to T cell-containing or T cell-depleted cultures of LDMNC induces the appearance of cells with the morphologic, phenotypic and functional properties of dendritic cells. Morphologically, the cells exhibit the long processes that are characteristic of dendritic cells. Phenotypically, the cells express high levels of class II MHC (HLA-DP), but are devoid of the markers of non-dendritic lineage (*e.g.* CD3, CD14 and CD16). Functionally, the cells are capable of inducing a strong proliferative response when used as stimulators in an allogeneic MLR, and are capable of presenting foreign antigen (*e.g.* tetanus toxoid) and thereby inducing an antigen-specific proliferation of autologous T cells.

Enrichment and culture of dendritic cells in DSCM is a novel and unexpected finding which becomes apparent when the T cell expansion activity of DSCM is suppressed. This dendritic cell-promoting activity of DSCM is useful in the generation and development of specific adoptive immunotherapies for cancer, infectious diseases and autoimmunity. For example, dendritic cells pulsed with tumor-associated antigen may be used as a vaccine or as a treatment to stimulate a patient's immune system against a tumor. Alternatively, antigen-pulsed dendritic cells may be used to stimulate MHC haplotype matched antigen-specific T cells *ex vivo* which, when re-infused into patients, provides enhanced anti-tumor activity. Similarly, dendritic cells pulsed with antigen derived from pathogenic organisms may be used as an *in vivo* therapeutic or prophylactic cellular vaccine, as a means of generating a therapeutic, as antigen-specific T cells *ex vivo*, as gene therapy vectors, or for fusion to tumor cells. Dendritic cells pulsed with protein or peptide antigen endocytose antigen, process the antigen and present antigenic fragments on class II MHC molecules. The resulting complex provides the stimulatory structure for activation of $CD4^+$ T lymphocytes. As an alternative to pulsing, dendritic cells of the same or a sufficiently close MHC haplotype to a patient may be transfected or transduced with a gene or genes encoding the antigen of interest in order to induce expression of the antigen in the cell. This strategy has the advantage that endogenous antigen synthesis is directed by the inserted DNA which leads to presentation of the antigen on class I MHC molecules. The resulting complex stimulates the $CD8^+$ arm of the immune response.

Production of Conditioned Medium

One embodiment of the invention is directed to methods for the production of compositions that promote the growth of antigen presenting cells, namely dendritic cells, macrophages and certain monocytes having this property. Growth can be defined as one or more of cell proliferation, the differentiation of antigen presenting cell progenitors, the maintenance of existing populations of specific antigen presenting cells and their progenitors, any promotion of activity along the pathway of hematopoiesis that leads to dendritic cells, monocytes, or macrophages or any combinations of these functions. These methods involve culturing cells with one or more biochemical agents that stimulate the secretion of a variety of factors that collectively favor the growth of the desired cells.

Cells that can be used as the starting cell population for the production of conditioned medium include cells of the lympho-hematopoietic system such as, for example, umbilical cord blood cells, adult peripheral blood cells, peripheral blood leukocytes, leukaphoresis blood cells, spleen cells, cells of the thymus or bone marrow, cells of the lymph nodes, primary and secondary lymphoid tissues including mucosa-associated lymphoid tissue (MALT), skin cells, or cells or cell lines derived from these populations. Successful culture can also be achieved using whole, unfractionated blood or buffy coat cells. Stimulation occurs by culturing the starting cell population in the presence of one or more biochemical agents. Preferably, these agents are mitogens, compounds that stimulate cell mitosis and/or cellular activation, and can be derived from prokaryotic sources (bacterial-derived), eukaryotic sources (plant-derived, mammalian-derived), or viral-derived sources. The process of cellular activation includes the events associated with differentiation such as the altered cell surface expression of receptors and other antigens, altered cytokine secretion, altered morphology and altered functional activity such as antigen-presenting activity. Preferred biochemical agents include phorbol esters such as, for example, phorbol-12-myristate-13-acetate (TPA), or related compounds such as, for example phorbol ester analogues such as diterpene esters (*e.g.* mezerein), ionophores such as calcium ionophores (*e.g.* A23187), and agents that induce activation and/or proliferation, all of which can be used either individually or in combination. Other related compounds

include, for example, phorbol (4-0-methyl) 12-myristate-13-acetate, phorbol (20-oxo-20-deoxy) 12-myristate-13-acetate, phorbol 12-monomyristate, phorbol 12, 13-didecanoate, phorbol 12,13-dibutyrate, phorbol 12,13-dibenzoate, and phorbol 12,13-diacetate. Cytokines such as interferon-alpha (IFNα), IFNβ and IFNγ can also be used in certain circumstances as well as stem cell factor (SCF), growth factors such as fibroblast growth factor (FGF) and the bone morphogenic proteins (BMPs). Other agents which can be used include other mitogenic plant-derived lectins such as concanavalin A (ConA), phytohemagglutinin (PHA) or pokeweed mitogen (PWM), bacterial mitogens such as, for example, streptolysin-S, bacterial toxins, super antigen, Staphylococcal enterotoxin A (SEA), Streptococcal protein A (SPA), galactose oxidase, or animal-derived agents such as, for example, monoclonal or polyclonal antibodies (*e.g.* OKT3, a T cell monoclonal antibody).

Culturing is performed under conventional conditions favorable to the starting or resulting cell population such as, for example, about 37°C, about 5% CO_2, and high humidity. Preferably, starting cells are cultured in the presence of one or more mitogenic agents for about two to twenty days, for about four to fourteen days, for about six to ten days, or for about four to twelve days. Incubation times vary according to cell number and cell density within the starting cell population. Upon completion of incubation, *i.e.* stimulation with mitogenic agents, medium may be separated from cells and used directly or stored for later use. Alternatively, as the activity of the conditioned medium of the invention is fairly stable, cells may be maintained in culture for longer periods of time without substantially altering the activity of the medium. Conditioned medium can be collected from these cells after at least one day following addition of mitogen, preferably after at least three days, more preferably after four days or even longer. As the conditioned medium of the invention can be produced from a variety of cells, cell cultures may comprise adherent or suspension cultures as appropriate or desired. Adherent cells may be preferred for certain applications as medium can be removed from cells with minimal effort.

Once the majority of cells have been separated, the medium may be further clarified by any one or combination of well-known methods such as, for example, sedimentation (*e.g.* Ficoll-Hypaque, a gravity-based density separation

medium), centrifugation or filtration to remove even more cells, cell debris and other particulate material from the medium. Centrifugation is preferably performed at about 500xg for about five minutes. However, increased centrifugation forces and time can be used as the active components of the medium are not significantly diminished. Filtration can also be used by passing the conditioned medium through one or more 0.45 or 0.22 μm filter membranes. Since many membranes tend to show an affinity for cell expression products, membranes are preferably pre-coated with a substance that has a high affinity for the membrane such as, for example, ovalbumin, human albumin, bovine serum albumin, casein, Tween, or other blocking agents. These substances are well known to stick tightly to membrane surfaces thereby allowing a larger percentage of the material of interest to pass through than would otherwise have been able.

Depending on the starting cell population and the stimulating agent or agents, the combination of factors that are present in DSCM can be varied in both concentration and content. Although the particular agents and cells used may vary, it is well within the skill of a cell biologist to determine the correct combination of cells with chemical agents that produces the conditioned medium of the invention. Further, as the results are easily determined (*i.e.* production of dendritic cells), to obtain conditioned medium of the invention, even by trial and error, is also well within the skill of those of ordinary skill in the art using the guidance provided herein.

Preferably, cells are stimulated by first incubation with one agent (for example mezerein) for a short period of time such as for example, less than twenty-four hours, preferably less than twelve hours and more preferably less than six hours. These cells are next cultured with a second agent (for example, concanavalin A) for a more extended period of time such as, for example, one or more days or one or more weeks. Culturing over extended periods of time may include supplementation of the medium with additional agents to maintain a constant concentration of active agent within the medium during incubation or to vary the stimulation received by the cells. For example, additional agent can be added when splitting or passing cell cultures. Accordingly, cells may be treated with agent(s) once, periodically or episodically as necessary or desired during incubation.

Dendritic Cell Selective Conditioned Medium

Another embodiment of the invention is directed to conditioned media produced according to the methods of the invention ("DSCM"). This medium is a rich source of both stimulatory and inhibitory factors possessing a wide range of hematopoietic activities. It is this combination of factors that produces the function of the conditioned medium of the invention.

As is known to those of ordinary skill in the art, cells cultured in the presence of one or more of these agents secrete a variety of soluble factors including, for example, many cytokines known to possess potent stimulatory and inhibitory effects on the growth and differentiation of lympho-hematopoietic cells (*e.g.* interleukin-2 (IL-2); granulocyte macrophage-colony stimulating factor (GM-CSF); tumor necrosis factor-α (TNF-α); macrophage inflammatory protein-α (MIP-1α). For example, conditioned medium produced from human umbilical cord blood has been measured to contain (concentration reported is the median measured concentration):
IL-8 (234 ng/ml) , TNF-β 112 ng/ml), MIP-1α (98 ng/ml), IL-2 (44 ng/ml), TGF-β1 (21 ng/ml), RANTES (15 ng/ml), MIP-1β (11 ng/ml), GM-CSF (11 ng/ml), TNF-RII (9.1 ng/ml), IL-1β (6.4 ng/ml), M-CSF (5.4 ng/ml), IL-13 (3.6 ng/ml), INF-γ (3.6 ng/ml), IL-1α (2.3 ng/ml), IL-16 (2.1 ng/ml). TNF-RI (1.8 ng/ml), Fas (1.3 ng/ml), TNF-α (0.37 ng/ml), IL-12 (0.26 ng/ml), SCF (0.20 ng/ml), IL-10 (0.02 ng/ml), IL-6 (0.007 ng/ml), IL-4 (0.006 ng/ml).

As compositions are produced by cells, the exact constituents of the composition may take a great deal of time, and expense, to identify fully. However, the specific components and the concentrations of those components within a DSCM composition can be determined by those of ordinary skill in the art. For example, compositions can be analyzed using HPLC, FPLC, quantitative ELISA using antibodies specific for known cytokines and many other techniques (*e.g. Current Protocols in Immunology*, ed. J.E. Coligan et al., Greene Publishing, New York, 1997; *Affinity Chromatography: A Practical Approach* P.D.G. Dean et al., IRL Press, Oxford, 1985; *Applications in HPLC in Biochemistry* A. Fallon et al, Elsevier Press, Amsterdam,

1987). Surprisingly, many compositions contain minimal or undetectable amounts of IL-4 (< 20 pg/ml). This is surprising as IL-4 was thought to be essential for dendritic cell development and, accordingly, an essential component of conventional compositions used for the culture of dendritic cells.

Conditioned medium of the invention may contain a small percentage of serum such as, for example, human serum or plasma or fetal calf serum that was used to culture the starting cell population. The concentration of serum in conditioned medium may reflect that used for culturing cells which is typically about 10% or less (*e.g.* 5%, 2% or 1%). However, conditioned medium of the invention may be supplemented with additional, fresh serum or plasma as desired. Accordingly, total serum concentrations in conditioned medium, which may include fractions of either or both depleted serum and fresh serum, can range from about 75% to 0%, preferably about 5% to about 50% and more preferably about 5% to 20%.

Compositions may further comprise an effective amount of one or more agents that suppress the proliferation, differentiation or maintenance of undesired cell types such as T cells or T cell progenitors. Such agents include, for example, anti-T cell antibodies (*e.g.* anti-CD3, anti-CD8, anti-thymocyte antibodies), cyclosporin, ATG, human umbilical cord plasma, human adult peripheral blood serum or plasma, agents that have a specificity for T cells or combinations of these agents. Compositions may also comprise chemical agents that stabilize the composition itself such as anti-oxidants, stabilizers or other agents to encourage cell growth (*e.g.* serum, cell growth factors, vitamins, amino acids, sugars, β-mercaptoethanol, human or bovine albumin, heparin, *etc.*).

Enrichment of Antigen Presenting Cells

Another embodiment of the invention is directed to a method for the enrichment of a biological sample for antigen presenting cells such as dendritic cells. Biological samples which can be enriched include samples containing an initial population of the desired antigen presenting cell type, such as dendritic cells or

progenitors of dendritic cells. Such samples include cells from lympho-hematopoietic sources such as human umbilical cord, adult peripheral blood, mucosa-associated lymphoid tissue (MALT), and other lymphoid or hematopoietic tissues. Tissues useful for the enrichment of dendritic cells include bone marrow, lymph node tissue, spleen, tonsils and skin. Preferably, the samples are obtained from a human source although most any mammalian source can be utilized. Samples may contain any number and variety of cells, and ideally contains a population of the desired type of antigen presenting cell that represents at least about one half of one percent of the total population of cells in the sample. Preferably, samples contain greater than about one percent, greater than about two percent, greater than about five percent or even greater than about ten percent. Of course, cell populations may contain substantially higher percentages of the desired type of such cells and may be pure populations of these cells as the compositions of the invention are also useful for maintaining antigen presenting cells in culture.

Enrichment comprises culturing a biological sample containing antigen presenting cells of the desired type in conditioned medium of the invention. Cells which proliferate include T cells and other cells of the hematopoietic system such as dendritic cells, monocytes, macrophages and other antigen-presenting cells. It is possible to alter culture conditions so as to favour the production of either dendritic cells, or monocytes and macrophages. Upon further culturing in the presence of agents that suppress T cell proliferation, dendritic cells and/or their progenitors become apparent and enriched in the culture. Enriched dendritic cells can also be maintained in compositions containing conditioned medium for long periods of time.

The method of the invention is directed to an activity contained in the conditioned medium, DSCM, which becomes apparent when the T cell expansion activity of the conditioned medium is suppressed, reduced or eliminated. When DSCM is incubated with unfractionated low density blood mononuclear cells (LDMNC), a large expansion of T lymphocytes is induced. The rapidity and extent to which this occurs masks other potential activities. However, when the T cell expansion activity of DSCM is suppressed, other activities become apparent. One such activity of DSCM enriches

dendritic cells and stimulates cell growth and differentiation. Another such activity of DSCM enriches the cell population for monocytes and macrophages and stimulates cell growth and differentiation.

Preferably, T cell population expansion is prevented by pre-incubation of the cell population under conditions that suppress, inhibit or delay T cell growth, but which promote survival of the desired type of antigen presenting cells and their progenitors. Many conditions which prevent T cell growth are known to those of ordinary skill in the art and include addition of a chemical or biological agent or agents to the culture medium that are cytotoxic or cytostatic to T cells. Such agents include, for example, anti-T cell antibodies which may be monoclonal or polyclonal, recombinant proteins or conjugated compounds, for example, toxins coupled (*e.g.* by conjugation, covalently or non-covalently linked, fusion proteins, *etc.*) to another compound that has a specific affinity for T cells, cyclosporin-A, ATG or combinations of these agents. Whether an agent is cytotoxic to T cell or cytostatic often depends on the concentration of the agent added to the culture medium.

Alternatively, suppression of T cell proliferation in response to DSCM may also be achieved by depletion of the starting cell population of T lymphocytes prior to the addition of DSCM. Methods for T lymphocyte depletion include affinity depletion techniques utilizing specific anti-T lymphocyte antibodies (*e.g.* anti-CD3, anti-CD8 or others) followed with physical, chemical or biological methods for removing the antibody-coated T cells (*e.g.* immuno-magnetic beads, chromatography matrices, biopanning flasks, complement-mediated lysis, rosetting and fluorescence-activated cell sorting; FACS). Cultures may also be depleted of T cells using non-antibody dependent techniques such as, for example, lectin-mediated affinity procedures, use of cyclosporin A, or physical separation methods such as differential density gradient centrifugation, centrifugal elutriation or combinations of these methods. Preferably, T cell growth is suppressed by prolonged incubation of the cell culture in the presence of umbilical cord blood plasma.

It has been discovered in accordance with one embodiment of the present

invention that plasma suppresses T cell growth in culture when used at concentration of from about 1% to about 75%. Incubation of a mixed cell population with plasma within this concentration range results in a gradual depletion of T cells in the population over time. Incubation times can range from about one day to about three days, about five days, about one week, about two to three weeks, or even one or more months as plasma does not otherwise interfere with growth of dendritic cells. Plasma concentrations which can be used include concentrations of from about 1% to about 50%, preferably from about 2% to about 35%, and more preferably from about 5% to about 20%. Other sources of plasma, such as adult peripheral blood plasma, plasma from other species or serum from clotted human or animal derived blood may also be used.

It has been discovered that anti-IL-2 antibodies suppress T-cell growth in culture. The starting population may be precultured with anti-IL-2 antibody prior to changing culture conditions by the addition of additional material such as DSCM, or anti-IL-2 antibody may be added at the same time as other materials, such as DSCM. Where the starting population is precultured in the presence of anti-IL-2 antibodies, it is preferable to add further anti-IL-2 antibodies when culture conditions are changed. The addition of anti-IL-2 antibodies to the preculture and culture medium increases the dendritic cell : T-cell ratio as compared to the use of plasma alone as a T-cell suppressing agent.

In certain embodiments, it is useful to suppress or inhibit other cell types. For example, in processes directed to the production of dendritic cells, monocytes and macrophages may be suppressed by use of a neutralizing antibody to macrophage colony stimulating factor (M-CSF).

Although DSCM is useful for the growth of dendritic cells, T cells (both naive and antigen-specific cells), monocytes and macrophages can also be cultured according to the methods of the invention. These cells can be co-cultured with dendritic cells, selectively enriched, or isolated and cultured as pure or nearly pure populations of cells.

Dendritic Cells

Another embodiment of the invention is directed to antigen presenting cells, such as dendritic cells, produced according to the methods of the invention. Dendritic cells of the invention possess one or more of the biological or biochemical characteristics associated with dendritic cells. Known characteristics of dendritic cells include expression of CD83, the dendritic cell marker, a distinct morphological appearance, and the ability to present antigen and to activate other cells such as T cells. Dendritic cells are also myeloperoxidase negative, $CD3^-$, $CD8^-$, $CD14^-$, $CD16^-$, and possess low levels of 5'-nucleotidase, non-specific esterase and cathepsin B, and express high levels of HLA class I^+ and class II^+ antigens. The phrase "dendritic cell" as used herein is intended to include both mature, fully differentiated dendritic cells as well as dendritic cell progenitors. Progenitors include cells committed to becoming dendritic cells or simply cells in the hematopoietic pathway that have the potential of becoming dendritic cells.

Dendritic cells, prepared according to the methods of the invention, can be maintained in tissue culture for extended periods of time at densities from about 10^3 cells/ml to about 10^6 cells/ml. At these higher densities, cells can be maintained in culture or preserved by freezing for later use in, for example, liquid nitrogen. Freezing can be accomplished using conventional methods of cryopreservation presently used for many types of mammalian cells. Cryopreserved dendritic cells may be maintained for long periods of time and thawed for proliferation or immediate use when needed.

Utilization of Antigen Presenting Cell Populations

Another embodiment of the invention is directed to methods for use of antigen presenting cells produced according to the methods of the invention. Specific uses for such cells include adoptive immuno-therapy, the creation of cellular vaccines and gene therapy for the treatment or prevention of a wide variety of disorders. For example, dendritic cells produced by the methods of the invention can be treated with antigen *ex vivo* and transferred into a patient to stimulate or modulate the patient's own

T cells *in vivo*. Alternatively, dendritic cells may be pulsed with antigen *in vitro* and used to expand specific populations of T cells *ex vivo*. T cells produced by these methods can be infused into a patient for the purpose of treating a particular disease or disorder.

Dendritic cells may be pulsed with antigen in the form of proteins, peptides or fragments thereof, or may be transfected or transduced with DNA or RNA encoding the gene for a specific antigen such as, for example, a foreign antigen or a tumor-associated antigen. The former method of introducing exogenous protein or peptide antigen into dendritic cells results in presentation of the antigen in association with class II MHC on cell surfaces which leads to stimulation of $CD4^+$ T cells. The latter method of gene transfer results in endogenous synthesis of the antigen with subsequent presentation of the antigen in association with class I MHC which leads to stimulation of $CD8^+$ T cells.

Physical methods for transfecting cells include the use of electroporation, osmotic shock, cationic lipids, DEAE-dextran, calcium phosphate precipitation, bead transfection and the biolistic particle gun. In addition, viral methods of transduction, for example using adenovirus, adeno-associated virus or retroviruses, may be used. Endogenous expression of peptides can also be mediated by the uptake of RNA messages added to dendritic cells exogenously. Another method for achieving antigen presentation of tumor antigens by dendritic cells involves fusion of the tumor cells directly to the dendritic cells. Cell fusion may be performed using physical methods such as polyethylene glycol-mediated membrane fusion or electrofusion. Fused dendritic - tumour cells may be used to activate T-cells *in vitro* prior to introduction of the activated T-cells into a patient, Alternatively, fused dendritic - tumour cells may be introduced into the patient to activate T-cells *in vitro*.

Further, dendritic cells that have been modified such that they no longer express co-stimulatory molecules are useful in modulating immune responses *in vivo*, thus providing a means of controlling or ameliorating autoimmune conditions.

Antigen presenting cells may also be used in gene therapy to express a desired genetic element in a host cell. Genetic elements can be transfected or otherwise introduced into antigen presenting cells or antigen presenting cell progenitors of the invention. Expression may be related to a disorder wherein there is an existing defect or deficiency in the expression of a gene. Administration of antigen presenting cells containing the genetic element that is capable of expression in the host can alleviate one or more symptoms or overcome the disorder. Expression can be regulated by regulating one or more genetic controls associated with the element (*i.e.* origins of replication, transcription or translation initiation sites or promoters, ribosome binding sites, transcription or translation terminators), or the number or placement of cells introduced to the host.

Disorders which can be treated with cellular vaccines include any disease associated with a foreign antigen. Such diseases include certain cancers (which possess cancer-related antigens), infectious diseases such as viral, bacterial and parasitic infections, and other disorders having an aberrantly expressed or undesired non-self antigen or stress proteins such as heat shock proteins. Such disorders can also be treated prophylactically as the dendritic cells and macrophages produced are antigen presenting cells and can be used to enhance an immune system later attacked by that foreign antigen or infectious agent.

Adoptive immunotherapy is the treatment of disease using cells of the immune system that have been activated or expanded *ex vivo*. Such modified cells are derived from patients and used to supplement, enhance, replace or otherwise modify their own inadequate or inappropriate immune response. Dendritic cells can also be used for adoptive immunotherapy. In adoptive immunotherapy, patients unable to clear infections may be treated with their own dendritic cells that have been modified *ex vivo* (*e.g.* incubated with antigen) to present antigens derived from the invading microorganism. Once infused, antigen-pulsed dendritic cells would stimulate patient T cells to attack and clear the infected cells. Alternatively, antigen-pulsed dendritic cells may be used *ex vivo* to generate large numbers of autologous antigen-specific T cells for re-infusion into the patient. Once infused, the T cells would attack and clear infected

cells and thereby cure the disease. Further, as only dendritic cells can stimulate naive T cells, dendritic cells pulsed with designated antigens could be used as prophylactic vaccines to stimulate cellular immunity and prevent the initial infections.

Certain tumors are known to express distinguishing antigens that are not normally found on healthy tissue. Such tumor-associated antigens (TAA) can be exploited for use in the adoptive immunotherapy of cancer. As in the treatment of infectious disease, dendritic cells pulsed with TAA could be infused into patients to stimulate a tumor-specific T cell response. Alternatively, the TAA-pulsed dendritic cells could be used to generate large numbers of tumor-specific T cells *ex vivo* which could be then infused into the patient.

Another advantage of antigen presenting cell therapy, especially antigen presenting cell gene therapy, is that antigen presenting cells can be easily introduced into a host animal. Cells that are introduced can be very closely screened and regulated for possible contamination. Such cells can be treated with agents and/or by processes that destroy the introduced cells subsequent to their desired effect. For example, antigen presenting cells of the invention that have been transfected with a genetic element may be irradiated prior to administration to the host. Radiation treatment can be regulated so as to allow for gene expression, but prevent genomic replication and, thus, any expansion of the administered cells. Alternatively, suicide genes such as the herpes simplex virus (HSV) thymidine kinase (TK) gene may be incorporated into cells and the cells induced to undergo apoptosis. For example, cells expressing TK can be specifically targeted and killed using nucleoside analogs such as ganciclovir, acyclovir or famciclovir. These techniques can be especially useful to meet FDA guidelines with regard to the testing of recombinant technology.

The following examples illustrate embodiments of the invention, but should not be viewed as limiting the scope of the invention.

Examples

Example 1 Preparation of DSCM.

Human umbilical cord blood containing 20 units of heparin per ml was used as the starting material for the preparation of DSCM. A sample of this blood was diluted 1:20 with 2% acetic acid and the total number of nucleated cells determined using a hemocytometer. The average number of nucleated cells per ml of human umbilical cord blood is 1.2×10^7. The average number of nucleated cells per umbilical cord is 6.0×10^8.

Blood was diluted in AIM-V™ media containing 20 units/ml heparin and 50 μM 2-mercaptoethanol (serum-free media: HCBM-2), to give a final concentration of 4×10^6 nucleated cells per ml. Mezerein was added, at a final concentration of 10 ng/ml and the mixture was incubated for 2 hours in a humidified incubator kept at 37°C and 5% CO_2. Concanavalin A was then added to a final concentration of 20 μg/ml and the incubation was continued under the same conditions for four days. Supernatant (DSCM) was harvested by centrifugation of the mixture at 500 x g for 30 minutes at 4°C. DSCM was stored at -20°C. Prior to use, the DSCM was thawed at room temperature or at 37 °C and clarified by centrifugation at 500 x g for 15 minutes at 4°C, followed by filtration using a 0.22 μm syringe-mounted filter.

Example 2 Culture of Antigen Presenting Cells - Suppression of the T Cell Expansion Activity of DSCM by Prolonged Culture of LDMNC with Umbilical Cord Blood Plasma.

Low density mononuclear cells (LDMNC) were prepared by density gradient centrifugation of adult peripheral blood using Ficoll-Hypaque™ (density = 1.077 g/ml). A volume of 15 ml of whole blood was layered onto an equal volume of Ficoll-Hypaque™ in a 50 ml conical tissue culture tube, which was then centrifuged at 400 x g for 30 minutes at room temperature. Interphase material containing the mononuclear cells was collected, and the cells were washed twice in HCBM-2 by centrifugation at 100 x g for 10 minutes at room temperature. Cells were diluted in HCBM-2 containing 10% fetal calf serum and incubated in polystyrene tissue culture

flasks overnight at 37°C and 5% CO_2. Alternatively, human serum or plasma, which does not contain foreign bovine antigens, can be substituted for fetal calf serum. The next morning, cells were washed twice by centrifugation and resuspended in HCBM-2. A sample of the cell suspension was diluted 1:20 with 2% acetic acid and the total number of nucleated cells determined using a hemocytometer. The average number of nucleated cells per ml of adult peripheral blood is 5.6×10^6, and the average yield of LDMNC per ml of adult peripheral blood is 1.2×10^6.

LDMNC were seeded into culture at $1\text{-}10 \times 10^5$ cells/ml in HCBM-2 containing 0 - 50% umbilical cord blood plasma. Cultures were incubated for 0 - 31 days at 37°C and 5% CO_2. At various time points, DSCM was added to a final concentration of 5%. One to three days after the addition of DSCM, the appearance of dendritic cells in the culture was observed, based on morphology.

Prior to the addition of DSCM, cultures contained large numbers of progenitor cells roughly globular in shape but with a very irregular perimeter. Upon treatment with DSCM, these cells were converted to very long, thin cells bearing the elongated processes morphologically characteristic of activated dendritic cells (Figure 1).

At least 12 days of pre-incubation of LDMNC with umbilical cord blood plasma was required for sufficient suppression of T cell expansion to permit the appearance of dendritic cells upon the addition of DSCM to the culture. Pre-incubation of LDMNC with umbilical cord blood plasma for periods of longer than 12 days further increased suppression of T cell expansion. This also tended to promote greater monocyte and macrophage growth at the expense of dendritic cells. Umbilical cord blood plasma at a concentration of 5% inhibited T cell expansion sufficiently such that dendritic cells were readily apparent in response to DSCM, provided the pre-incubation of LDMNC with the plasma was of at least 12 days duration. Increasing concentrations of umbilical cord blood plasma further increased suppression of T cell expansion. However, this also tended to promote greater monocyte and macrophage growth. The number of dendritic cells that appeared in the culture in response to the addition of

DSCM was dependent on the number of LDMNC seeded into the culture. The yield of dendritic cells increased with increasing seeding density from 1 x 10^5 to 5 x 10^5 LDMNC per ml. Above this seeding density, monocytes and macrophages tended to take over and dominate the cultures. Optimal conditions for obtaining dendritic cells, based on morphology for these specific cells, were determined to be as follows:

3.5 x 10^5 LDMNC + 5 - 20% umbilical cord blood plasma

↓ 12 days, 37°C, 5% CO_2

+ 5% DSCM

↓ 1 day, 37°C, 5% CO_2

dendritic cells

The production of monocytes and macrophages in LDMNC cultures following treatment with DSCM was especially favored in situations where the LDMNC culture which was pretreated with umbilical cord plasma was seeded at a density greater than 5 x 10^5 LDMNC per ml, was treated with umbilical cord plasma at concentrations in excess of 10 %, or was preincubated with umbilical cord blood plasma for greater than 15 days.

Example 3 Culture of Dendritic Cells - Depletion of T Cells Prior to Culture.

Suppression of T cell expansion activity of DSCM may be further enhanced by depletion of T cells from the LDMNC population prior to culture. Adult peripheral blood LDMNC were depleted of $CD3^+$ cells by affinity depletion using specific antibody and magnetic beads (StemSep™ Lineage Depletion and Cell Purging for Human Hematopoietic Cells - Stem Cell Technologies Inc.; Vancouver, BC). Efficiency of the depletion is shown in Table 1. T cell-depleted LDMNC were seeded into culture at 3.5 x 10^5 cells/ml in HCBM-2 containing 5% or 20% umbilical cord blood plasma. The culture was incubated for 4 days at 37°C and 5% CO_2 at which time DSCM was added to a final concentration of 5%. Consistent with previous observations,

the addition of DSCM to T cell-suppressed cultures induced the appearance of cells with distinct dendritic morphology. By depleting LDMNC of T cells prior to culture it was possible to shorten the umbilical cord blood plasma pre-incubation to 4 days, with the result that the number of T cells, monocytes and macrophages in the cultures decreased, and the reproducibility of obtaining the dendritic cells increased.

Table 1
Depletion of $CD3^+$ T Cells from LDMNC

Average % $CD3^+$ T Cells (prior to depletion):	54%	(+/- 18%)	n = 7
Average % $CD3^+$ T Cells (after depletion):	5%	(+/- 4%)	n = 7
Average Yield of $CD3^+$ Depleted Cells:	32%	(+/- 19%)	n = 17

Example 4 Culture of Dendritic Cells - Suppression of T-Cell Expansion Activity of DSCM by the addition of anti-IL-2 antibody during culture

Adult peripheral blood LDMNC were depleted of CD3+ cells by affinity depletion as described in Example 3. The CD3+ depleted LDMNC were seeded into culture at 3.5×10^5 cells/ml in HCBM-2 containing 5 % umbilical cord plasma. The culture was incubated for 3 days at 37° C and 5 % CO_2.

After five days, DSCM was added to a final concentration of 5 %, and mouse anti-human IL-2 monoclonal antibody was added to a final concentration of 0, 2, or 5 μg/ml. The cultures were incubated at 37°C and 5 % CO_2 for four or six days, at which times cells were counted and analyzed by flow cytometry for surface expression of CD83 and CD3. Dendritic cells were defined by the expression of CD83 in the absence of co-

expression of CD3. The results are shown in Table 2, below.

The addition of DSCM induced an increase in cell count, an increase in the number of dendritic cells and an increase in the number of T-cells. The increase in the total cell count and total number of T-cells was blocked by the addition of anti-IL-2 antibody; however, the increase in the number of dendritic cells was not blocked by the addition of anti-IL-2 antibody, although the extent of the increase was reduced.. Thus, anti-IL-2 antibody is effective in suppressing T-cell expansion in culture, while allowing the expansion of a population of dendritic cells.

Table 2
Suppression of T-Cell Expansion Activity of DSCM by the Addition of anti-IL-2 Antibody During Culture

Condition	4 days post DSCM + aIL2 addition			6 days post DSCM + aIL2 addition		
	count	%DC	%T	count	%DC	%T
P	8.7e4	5.9	22.6	9.6e4	5.8	23.4
P+DSCM	1.9e5	40.7	19.1	3.5e5	20.0	58.1
P+DSCM+ 2 ug/ml aIL-2	8.4e4	35.6	16.5	7.5e4	37.9	28.3
P+DSCM+ 5 ug/ml aIL-2	9.1e4	35.7	14.6	7.6e4	37.8	23.0

Condition	4 days post DSCM + aIL2 addition			6 days post DSCM + aIL2 addition		
	total cell count	DC* count	T** count	total cell count	DC* count	T** count
P	8.7e4	5133	19662	9.6e4	5568	22464
P+DSCM	1.9e5	77330	36290	3.5e5	70000	203350
P+DSCM+ 2 ug/ml aIL-2	8.4e4	29904	13860	7.5e4	28425	21225
P+DSCM+ 5 ug/ml aIL-2	9.1e4	32487	13286	7.6e4	28728	17480

* DC count = total cell count x %DC
**T count = total cell count x %T

Example 5 Phenotypic Analysis of the Dendritic Cells.

The phenotype of the dendritic cells was analyzed by immunohistochemical staining using antibodies specific for well characterized cell surface markers and by cytochemical staining using colorimetric substrates for cellular enzymes. For this purpose, cultures of dendritic cells were prepared in the flat-bottom wells of 24-well tissue culture plates (CoStar; Cambridge, MA) as described in Examples 2 and 3. In preparation for immunohistochemical staining, the wells were emptied and the cells were fixed onto the plastic surface by treatment with 2% formaldehyde/0.05% gluteraldehyde for 10 minutes at room temperature. Fixative was removed and the cells were washed twice with phosphate buffered saline (PBS). Non-specific binding of immunoglobulin was blocked by incubation with PBS containing 2% normal goat serum (2% NGS/PBS) for 15 minutes at 37°C. Cells were incubated with 10 μg of primary antibody in 0.5 ml of 2% NGS/PBS for one hour at 37°C. Following this step, wells were emptied of antibody and washed three times with 0.5% NGS/PBS. Binding of the primary antibody to the cells was detected by subsequent incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (GaMIgG-HRP - Caltag Laboratories; Burlingame, CA) diluted 1:100 with 2% NGS/PBS. After a final wash, color development was accomplished using 3-amino-9-ethylcarbazole and hydrogen peroxide (AEC staining kit; Sigma Chemical Co.; St. Louis, MO).

For cytochemical staining, commercially available kits for the detection of myeloperoxidase (MPX) and non-specific esterase (NSE) (Sigma Chemical Co.; St. Louis, MO) were used. As described in Table 3, phenotype of the cells was consistent with the phenotype of mature dendritic cells as described in the scientific literature.

Table 3
Phenotype of Dendritic Cells

	Dendritic Cells (prepared according to the present method)	Dendritic Cells (according to the literature)
Class II MHC	+++	+++
CD3	-	-

CD14	-	-
CD16	-	-
MPX	-	-
NSE	- or -/+	- or -/+

Example 6 Expression of CD 83.

CD83 is a 45 kD glycoprotein which has been described recently as a differentiation antigen specific for dendritic cells. CD83 antigen is not detected on the surface of freshly isolated dendritic cells, but becomes detectable after culture or maturation (Blood 89:3708, 1997). Cultures of dendritic cells were prepared in the flat bottom wells of 24-well tissue culture plates (CoStar; Cambridge, MA) as described in Example 3. One day following the addition of DSCM to the cultures, dendritic cells were fixed and stained by immunohistochemistry, as described in Example 3, using a primary antibody specific for CD83 (Immunotech Inc.; Westbrook, ME). Additional cultures of dendritic cells were maintained in umbilical cord plasma and DSCM. After four days, the dendritic cells had undergone a substantial morphological change from long, thin, adherent cells to rounder, less adherent cells that occurred in clusters and thus, appeared to be proliferating.

Although the long, thin adherent cells (one day post-DSCM) did not express CD83, the rounder, less adherent cells (four days post-DSCM) highly expressed CD83. This result was confirmed by flow cytometry using the same primary anti-CD83 antibody followed by phycoerythrin-labeled goat anti-mouse IgG (Figure 2). The $CD83^+$ cells co-expressed HLA-DP (class II MHC), but not CD3 or CD14, consistent with the expected phenotype of dendritic cells. Quantitatively, DSCM induced a 16-fold increase in the number of $CD83^+$ cells over a period of four days (Table 3). Thus, DSCM induces the enrichment of dendritic cells *ex vivo*.

Table 4
Expression of CD83

	Before Treatment with DSCM	After Treatment with DSCM
Cell Count	2.2×10^5/ml	3.75×10^5/ml

Percent $CD83^+$	1.6 %	15.3 %
Total $CD83^+$	3.52×10^3	5.73×10^4 (16-fold increase)

Example 7 Functional Analysis of Dendritic Cells - Mixed Leukocyte Reaction.

Dendritic cells are considered to be the most potent of all of the cells of the lympho-hematopoietic system in terms of their ability to stimulate proliferation of allogeneic responder T cells in the mixed leukocyte reaction (MLR).

Cultures of dendritic cells were prepared in the flat-bottom wells of 96-well tissue culture plates as described in Example 3. Wells contained no dendritic cells, dendritic cells generated using 5% umbilical cord blood plasma and DSCM, or dendritic cells generated using 20% umbilical cord blood plasma and DSCM. Media, plasma and DSCM were removed from the dendritic cells and replaced with fresh medium containing 10% umbilical cord blood plasma and no responder cells, autologous responder cells - *i.e.* LDMNC from the same individual from whom the dendritic cells were derived (self), or allogeneic responder cells - *i.e.* LDMNC from a different, HLA-mismatched individual (non-self). Cell proliferation was measured by ^{3}H-thymidine uptake: the MLR was allowed to proceed for 6 days at 37°C and 5% CO_2 with 1 μCi per well of ^{3}H-thymidine present during the last 18 hours. Wells were harvested using a Skatron cell harvester (Skatron Instruments Inc.; Sterling, VA) and the number of cpm of ^{3}H-thymidine incorporated into DNA were measured using a β-scintillation counter (Figure 3). Very little proliferation occurred when autologous, HLA-matched responder cells were incubated with the dendritic cells. However, when allogeneic, HLA-mismatched responder cells were incubated with dendritic cells, substantial proliferation occurred as indicated by the incorporation of ^{3}H-thymidine into DNA. Very little proliferation of either autologous or allogeneic responder cells occurred in the absence of dendritic cells. These results indicate that the DSCM-generated dendritic cells are potent stimulators of the allogeneic MLR.

Example 8 Functional Analysis of the Dendritic Cells - Antigen Presentation.

As professional antigen presenting cells (APC), dendritic cells are able to take up foreign antigen, process it into peptide fragments and present the peptide fragments in association with MHC molecules to autologous T cells. Cultures of dendritic cells were prepared in the flat-bottom wells of 96-well tissue culture plates (CoStar; Cambridge, MA) as described in Example 3. Wells contained either no dendritic cells or dendritic cells generated using 20% umbilical cord blood plasma and DSCM. Medium, plasma and DSCM were removed and replaced with fresh medium containing 10% umbilical cord blood plasma, foreign antigen (tetanus toxoid - 0, 0.2 or 1 μg/ml) and autologous responder cells - *i.e.* LDMNC from the same individual from whom the dendritic cells were derived. Cell proliferation was measured after 4 days, using ^{3}H-thymidine uptake as described in Example 7 (Figure 4a). Cell proliferation occurred only in the presence of all three components required for antigen presentation - *i.e.* dendritic cells (APC), responder cells (T lymphocytes) and foreign antigen (tetanus toxoid). These results indicate that DSCM-generated dendritic cells are capable of presenting foreign antigen to autologous T cells.

The above experiment was repeated with some modifications: dendritic cells were generated using 5% umbilical cord blood plasma instead of 20%, higher concentrations of tetanus toxoid were used (0, 1, 10 or 100 μg/ml instead of 0. 0.2 and 1 μg/ml), autologous responder cells were purified T cells instead of LDMNC, and the response was measured at 5 days instead of 4 days (Figure 4b). Results were entirely consistent with the previous finding that the DSCM-generated dendritic cells are able to process and present foreign antigen to autologous T cells.

Example 9 DSCM-Induced Differentiation of multi-potential progenitors expanded using defined cytokines

Human umbilical cord blood LDMNC were prepared as described in Example 2 and were enriched for CD34$^+$ multi-potential hematopoietic progenitor cells by affinity depletion using a cocktail of antibodies specific for mature lineage committed cells (anti-CD2, anti-CD3, anti-CD14, anti-CD16, anti-CD19, and anti-CD24, anti-CD56, anti-CD66b and anti-glycophorin A) and immuno-magnetic beads (Stem Cell

Technologies, Vancouver, BC). The enriched population was seeded into culture at 1 - 2 x 10^4 cells/ml in high growth factor medium (IMDM - Iscove's Modified Dulbecco's Medium containing 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin (IMDM-S), supplemented with 100 ng/ml IL-3 and 100 ng/ml SCF). Under these conditions, the cells underwent a 1000-8000 fold expansion over a time period of three-five weeks. During this expansion phase, the maintenance of multi-potential progenitor cells capable of giving rise to cells of the granulocyte and monocyte/macrophage lineages (CFU-GM) was demonstrated using a colony forming assay in methylcellulose (Stem Cell Technologies, Vancouver, BC). The starting number of CFU-GMs in the CD34-enriched population was approximately 30 CFU-GMs per 1 x 10^4 CD34-enriched cells, while the cultured cells generally produced 1-5 CFU-GMs per 1 x 10^5 cultured cells.

After the expansion phase, the cells were reseeded at 1 x 10^5 cells/ml in HCBM-2 supplemented with 5% DSCM and 5% umbilical cord blood plasma. This caused the cells to undergo a major morphological change from small, round, non-adherent cells to larger, more spread out, adherent cells, most of which appeared to be macrophages, but a significant number (5-10%) of which had the characteristic dendritic cell morphology described in Example 1 (Figure 1 - "after treatment"). When dislodged from the plastic tissue culture surface by incubation on ice and analyzed by flow cytometry, a corresponding number (5-10%) of the detached cells were found to be $CD83^+$. By contrast, when GM-CSF and IL-4 were substituted for DSCM and umbilical cord blood plasma, the differentiation that occurred tended to be exclusively in the direction of polymorphonuclear granulocytes and contained few, if any, $CD83^+$ cells.

These results indicate that DSCM can be used to induce the differentiation of *ex vivo* expanded multi-potential progenitor cells into dendritic cells and macrophages.

Other embodiments and uses of the invention will be apparent to those

skilled in the art from consideration of the specification and practice of the invention disclosed herein. All patents and patent applications, including application publication number WO 98/33891, and all other documents referenced herein, for whatever reason, are specifically incorporated by reference.

WE CLAIM:

1. A process for culturing preselected antigen presenting cell types or progenitors thereof, which comprises:

(a) culturing a starting cell population containing viable antigen presenting cells or progenitors thereof, in a culture medium containing a suitable quantity of conditioned medium "DSCM" which is derived from a aqueous medium previously used for culturing a population of cells of the lympho-hematopoietic system in the presence of at least one stimulatory biochemical agent, and

(b) obtaining in the product of step (a) a cell population containing an increased number or relative proportion of the preselected antigen presenting cells or progenitors thereof.

2. The process of claim 1 wherein the starting cell population contains viable antigen presenting cell progenitors which are multi-potential hematopoietic progenitor cells.

3. The process of claim 2 wherein the starting cell population is enriched for CD 34+ cells.

4. The process of any preceding claim wherein the viable antigen presenting cell type or progenitors thereof constitute at least one-half of one percent of the total starting cell population.

5. The process of any of claims 1 - 3 wherein the viable antigen presenting cell type or progenitors thereof constitute at least five percent of the total starting cell population.

6. The process of any preceding claim wherein the preselected antigen presenting cell type is dendritic cells.

7. The process of any of claims 1 - 5 wherein the preselected antigen presenting

cell type is comprised of monocytes and macrophages.

8. The process of any of claims 1 - 5 wherein the preselected antigen presenting cell type is macrophages.

9. The process of any preceding claim wherein said stimulatory biochemical agent is a mitogen.

10. The process of claim 9 wherein said mitogen is a plant-derived lectin, a phorbol ester, a phorbol ester analogue, or a calcium ionophore.

11. The process of claim 9 wherein said mitogen is concanavalin A or mezerein.

12. The process of any of claims 1 - 8 wherein the stimulatory biochemical agents employed are concanavalin A and mezerein.

13. The process of any preceding claim wherein the conditioned medium further contains a compound effective to suppress, delay, or inhibit growth of undesired cell types.

14. The process of claim 13 wherein said compound is effective against T-cell growth.

15. The process of claim 13 wherein said compound is serum or plasma.

16. The process of claim 15 wherein said serum or plasma is human umbilical cord serum or plasma.

17. The process of claims 15 or 16 wherein the serum or plasma constitutes from 1 - 50 % of total culture medium volume.

18. The process of claims 15 or 16 wherein the serum or plasma constitutes from 5

- 20 % of total culture medium volume.

19. The process of claim 14 wherein said compound is anti-IL-2 antibody.

20. The process of claim 14 wherein said compound is an immunosuppressive agent.

21. The process of any preceding claim wherein the DSCM is derived from an aqueous medium previously used for culturing human umbilical cord blood cells.

22. The process of any of claims 1 - 20 wherein the DSCM is derived from an aqueous medium previously used for culturing human peripheral leukocytes.

23. A composition of matter comprising a population of preselected antigen presenting cells produced according to the process of any preceding claim.

24. A composition of matter comprising the population of claim 21 being substantially free of viable cells of any other type.

25. The composition of claims 23 or 24 wherein the preselected antigen presenting cell population is a population of dendritic cells.

26. The composition of claims 23 or 24 wherein the antigen presenting cell population is a population of monocytes and macrophages.

27. The composition of claims 23 or 24 wherein the antigen presenting cell population is a population of macrophages.

28. A process of suppressing T-cell growth in culture comprising culturing a population of viable cells which includes T-cells or progenitors thereof in an aqueous culture medium which contains an effective amount of at least one T-

cell growth-suppressive compound selected from serum, plasma, anti-IL-2 antibody, and immunosuppressive agents.

29. The process of claim 28 wherein the T-cell growth suppressive compound is selected from serum or plasma.

30. The process of claim 28 wherein the T-cell growth suppressive compound is plasma.

31. The process of claims 29 or 30 wherein the serum or plasma is derived from human umbilical cord.

32. The process of claims 29 - 31 wherein the serum or plasma constitutes from 1 - 50 % of total culture medium volume.

33. The process of claims 29 - 31 wherein the serum or plasma constitutes from 5 - 20 % of total culture medium volume.

34. The process of claim 28 wherein the T-cell growth-suppressive compound is anti-IL-2 antibody.

35. The process of claim 28 wherein the T-cell growth suppressive compounds employed are anti-IL-2 antibody and plasma.

36. The process of claims 34 or 35 wherein anti-IL-2 antibody is added to a final concentration of between 2 - 5 μg/ml.

37. The process of claim 28 wherein the T-cell growth-suppressive compound is cyclosporin A.

38. A process of immunotherapy for a patient in need thereof in which antigen presenting cells having a MHC haplotype compatible with that of the patient,

and produced according to the process of any of claims 1 - 6 or 9 - 22, are treated with a desired antigen and subsequently introduced into the patient in order to stimulate or modulate the immune response of the patient's own lymphocytes.

39. A process a immunotherapy for a patient in need thereof in which antigen presenting cells having a MHC haplotype compatible with that of the patient, and produced according to the process of any of claims 1 - 6 or 9 - 22 are treated with a desired antigen and then cultured with specific populations of T-cells of a compatible MHC haplotype, followed by the introduction of the activated T-cells into the patient.

40. A process of immunotherapy for a patient in need thereof in which antigen presenting cells of having a MHC haplotype compatible with that of the patient, and produced according to the process of any of claims 1 - 6 or 9 - 22, are transfected with suitable nucleic acids encoding the gene for a specific antigen and subsequently introduced into a patient in order to stimulate or modulate the immune response of the patient's own lymphocytes.

41. A process of immunotherapy for a patient in need thereof in which antigen presenting cells having a MHC haplotype compatible with that of the patient, and produced according to the process of any of claims 1 - 6 or 9 - 22, are transfected with suitable nucleic acids encoding a gene for a specific antigen and then used to expand specific populations of T-cells of a compatible haplotype, followed by the introduction of the expanded T-cells into the patient.

42. A process of immunotherapy for a patient in need thereof in which antigen presenting cells having a MHC haplotype compatible with that of the patient, and produced according to the process of any of claims 1 - 6 or 9 - 22 are fused with tumour cells in order to induce the presentation of tumour-specific antigens on the cell surface, and subsequently introduced into a patient in order

to stimulate or modulate the immune response of the patient's own lymphocytes.

43. A process of immunotherapy for a patient in need thereof in which antigen presenting cells of having a MHC haplotype compatible with that of the patient, and produced according to the process of any of claims 1 - 6 or 9 - 22 are fused with tumour cells in order to induce the presentation of tumour-specific antigens on the cell surface and then used to expand T-cells of a compatible MHC haplotype, followed by the introduction of the expanded T-cells into the patient.

44. A process of gene therapy for a patient in need thereof in which antigen presenting cells having a MHC haplotype compatible with that of the patient, and produced according to the process of any of claims 1 - 6 or 9 - 22, are transfected with an expressable genetic element, followed by the introduction of the transfected antigen presenting cells into the patient.

45. The process of claim 44 wherein the genetic element introduced corresponds to a gene product which is insufficiently or aberrantly expressed in the patient.

46. A process of immunotherapy for a patient in need thereof whereby:

(a) macrophages or dendritic cells of having a MHC haplotype compatible with that of the patient, and produced according to the process of any of claims 1 - 6 or 9 - 22 are altered to prevent normal interactions between co-stimulatory molecules on the macrophage or dendritic cell and a T-cell, and

(b) the altered macrophages or dendritic cells are exposed to a desired antigen to induce antigen presentation, and

(c) the altered macrophages or dendritic cells are introduced into the patient.

47. The process of claim 46 wherein the macrophages or dendritic cells are

genetically altered.

48. The process of claim 46 wherein the macrophages or dendritic cells are altered by the insertion of a DNA sequence into a portion of a gene encoding a co-stimulatory molecule so as to reduce the functionality of the molecule.

49. The process of any of claims 46 - 48 wherein the co-stimulatory molecule is B7.1 or B7.2.

50. The process of any of claims 38 - 45 wherein the antigen presenting cells have the same MHC haplotype as the patient.

51. The process of any of claims 39, 41, 43 or 50 wherein the T-cells have the same MHC haplotype as the patient.

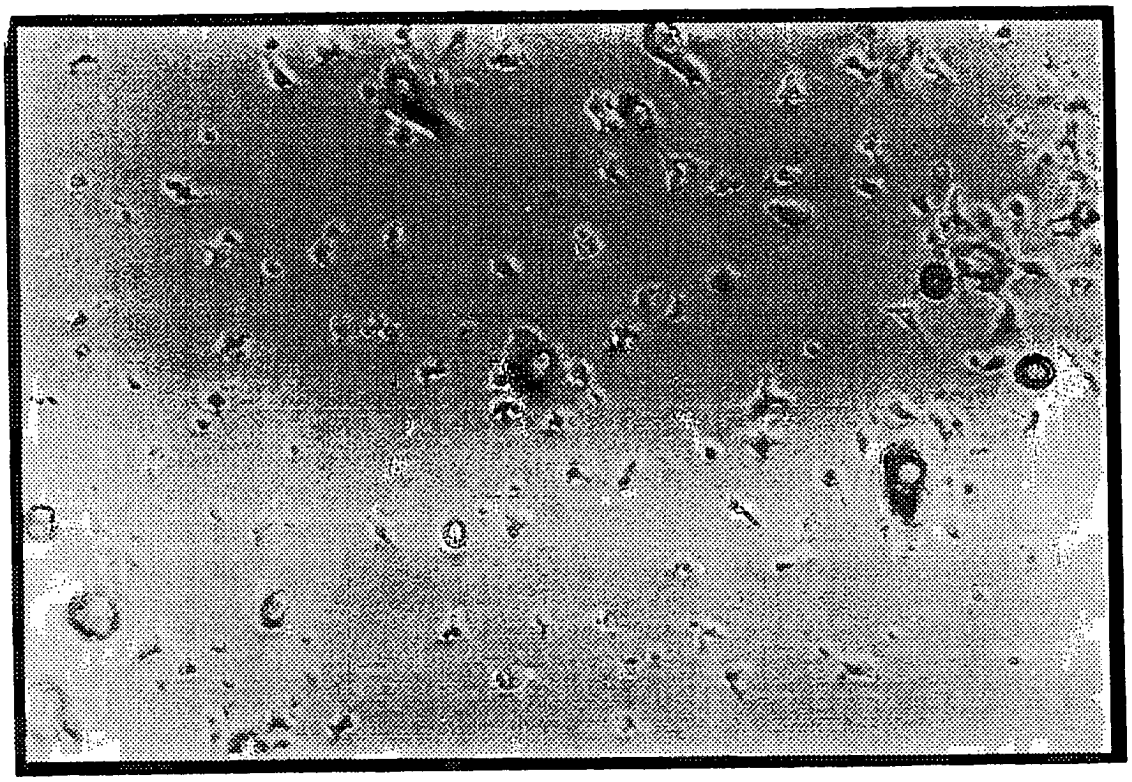
52. The process of claims 46 - 49 wherein the macrophages or dendritic cells have the same MHC haplotype as the patient.

53. The process of any of claims 38 - 45 or 50 - 51 wherein the antigen presenting cells are dendritic cells.

54. The process of any of claims 38 - 45 or 50 - 51 wherein the antigen presenting cells are macrophages.

Figure 1 **Dendritic Cell Culture**

before treatment:



after treatment:

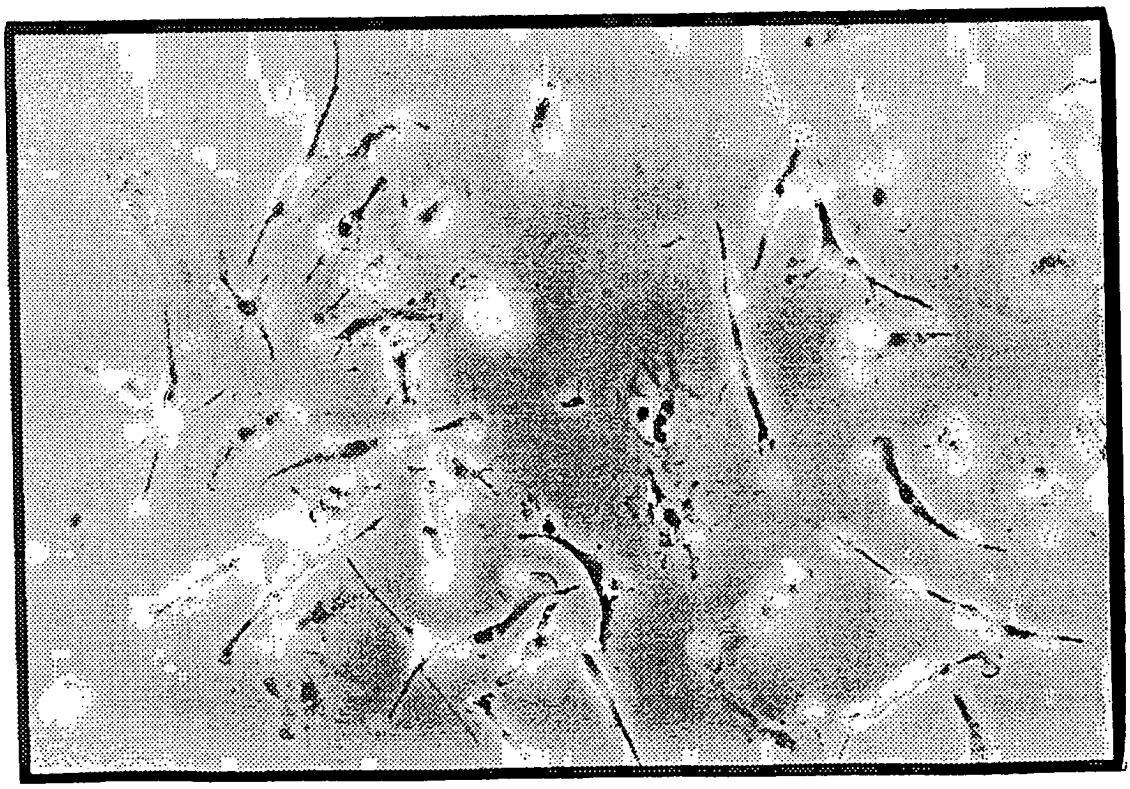


Figure 2

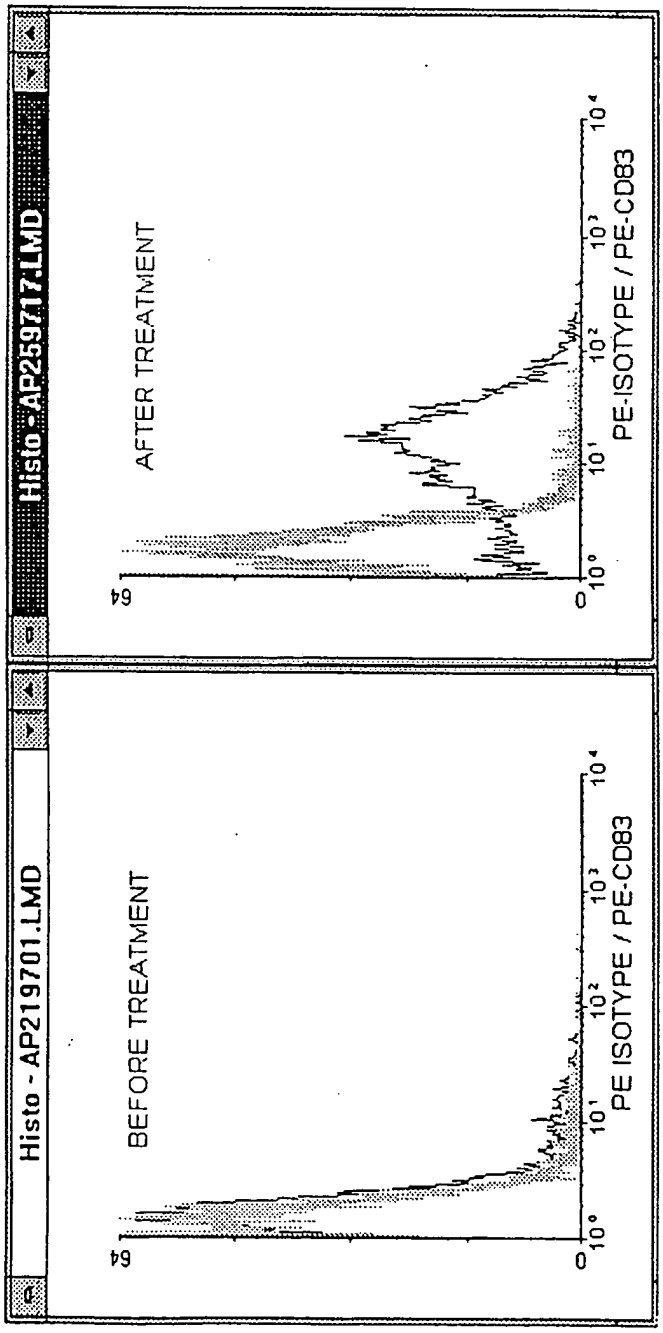

Histo - AP219701.LMD
BEFORE TREATMENT
PE ISOTYPE / PE-CD83
64
0
10°
10¹
10²
10³
10⁴
Histo - AP219717.LMD
AFTER TREATMENT
PE-ISOTYPE / PE-CD83
64
0
10°
10¹
10²
10³
10⁴

Figure 3

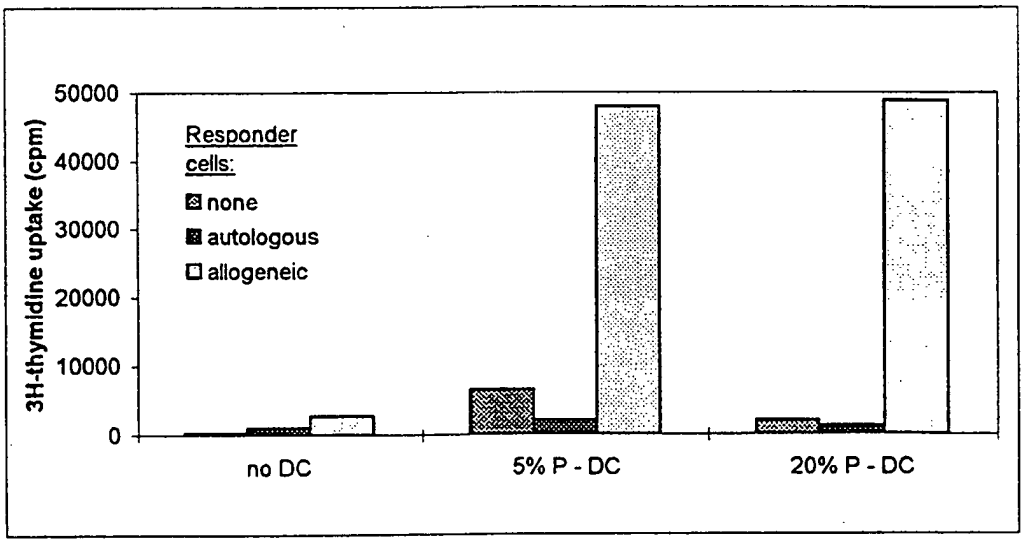
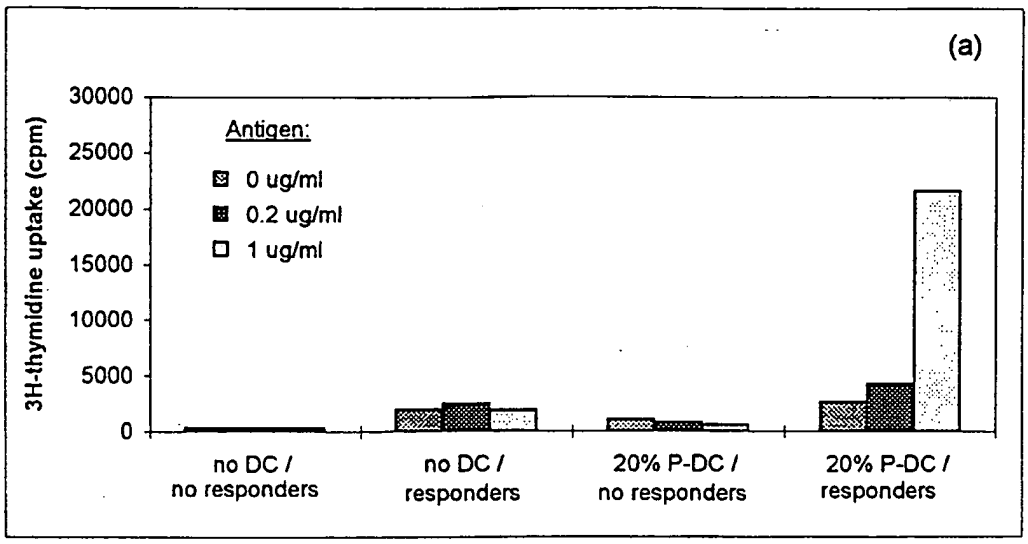
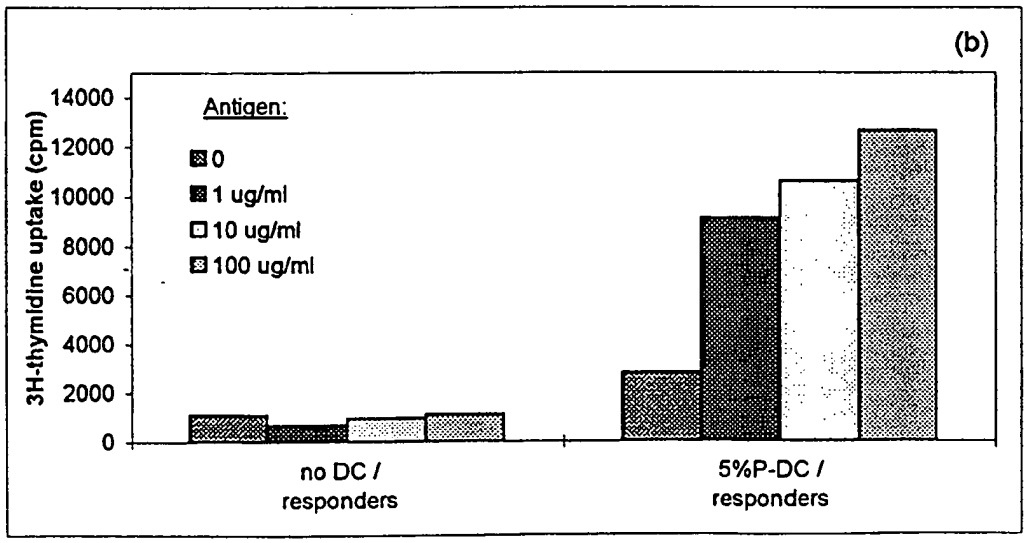

3H-thymidine uptake (cpm)
50000
40000
30000
20000
10000
0
Responder cells:
none
autologous
allogeneic
no DC
5% P - DC
20% P - DC

Figure 4


(a)
3H-thymidine uptake (cpm)
30000
25000
20000
15000
10000
5000
0
Antigen:
0 ug/ml
0.2 ug/ml
1 ug/ml
no DC / no responders
no DC / responders
20% P-DC / no responders
20% P-DC / responders


(b)
3H-thymidine uptake (cpm)
14000
12000
10000
8000
6000
4000
2000
0
Antigen:
0
1 ug/ml
10 ug/ml
100 ug/ml
no DC / responders
5%P-DC / responders

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/CA 98/01067

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 A61K35/14 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 29182 A (THE ROCKEFELLER UNIVERSITY) 14 August 1997 see the whole document ---	1-54
A	THURNHER M ET AL: "In vitro generation of CD83+ human blood dendritic cells for active tumor immunotherapy." EXPERIMENTAL HEMATOLOGY, (1997 MAR) 25 (3) 232-7, XP002100889 see the whole document ---	
A	REDDY A ET AL: "A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells." BLOOD, (1997 NOV 1) 90 (9) 3640-6, XP002100890 see the whole document ---	1,38-54
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 April 1999	06/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

1

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/CA 98/01067

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ³	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BENDER A ET AL: "IMPROVED METHODS FOR THE GENERATION OF DENDRITIC CELLS FROM NONPROLIFERATING PROGENITORS IN HUMAN BLOOD" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 196, 1996, pages 121-135, XP002034181 see the whole document	1-27

INTERNATIONAL SEARCH REPORT

...ternational application No.
PCT/CA 98/ 01067

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 38-54 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/CA 98/01067

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9729182 A	14-08-1997	AU 1955997 A	28-08-1997